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Research Article

A single tryptophan residue of endomannosidase is crucial for Golgi localization and *in vivo* activity

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Keywords. Endomannosidase, Golgi apparatus, endoplasmic reticulum, *N*-glycosylation, α 1-antitrypsin, CHO Lec 23 cells.

Introduction

Removal of glucose residues from asparagine-linked $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ oligosaccharides by trimming glucosidase I and II represents an important initial post-translational modification of *N*-glycans. It starts the pathway for further trimming and elongation reactions in the endoplasmic reticulum (ER) and subsequently in the Golgi apparatus yielding the spectrum of mature *N*-glycans [1, 2]. Glucose trimming by glucosidase II has an additional aspect since it is of importance for quality control of glycoprotein folding.

Here, glucosidase II-mediated de-glucosylation permits the exit of glycoproteins from the calnexin/calreticulin cycle [3–5].

An alternate glucosidase-independent pathway of glucose trimming by endo- α -endomannosidase was discovered by Spiro and colleagues [6, 7]. In contrast to the ER-resident glucosidase II [8, 9], endomannosidase is primarily located in the *cis* and medial Golgi apparatus [9]. It is a unique endoglycosidase and cleaves the α 1,2 linkage between the glucose-substituted mannose and the remainder mannose branch of *N*-glycans. The resulting $\text{Man}_8\text{GlcNAc}_2$ A-isomer is no longer a substrate for UDP-Glc:glycoprotein glucosyltransferase, but can be processed by Golgi-mannosidase I [2, 10, 11]. Endomannosidase carries

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out a substantial amount of de-glucosylation *in vivo*, as demonstrated by the processing of glycoproteins in HepG2 cells [12]. Furthermore, the occurrence of a Glc₃Man tetrasaccharide in the urine of patients with a human congenital disorder of glycosylation caused by glucosidase I deficiency might be an indication of the action of endomannosidase [13]. Besides mono-glucosylated oligosaccharides, di- and tri-glucosylated oligosaccharides are also processed by endomannosidase [6, 14, 15]. The latter two oligosaccharides typically occur in glucosidase II-deficient Phar2.7 cells [16] or in glucosidase I-deficient CHO Lec23 cells [17, 18], respectively. Endomannosidase also counteracts the effects of experimental glucosidase I and II inhibition by castanospermine [19, 20]. Thus, by circumventing either a glucosidase deficiency or inhibition, endomannosidase permits the formation of mature, complex type *N*-glycans. As recently demonstrated, glucosylated oligosaccharides of both wild-type and of misfolded variants of α 1-antitrypsin are processed by endomannosidase [21]. This indicates that the Golgi-localized endomannosidase provides a back-up mechanism for de-glucosylation of ER-escaped native and misfolded glycoproteins allowing the processing of their *N*-glycans to mature ones.

Endomannosidase activity is detectable in vertebrates tissues and in a range of animal and human cell lines [9, 15, 22, 23]. However, CHO and CHO Lec23 cells seem to be endomannosidase deficient. This is based on the lack of measurable enzyme activity by sensitive *in vitro* assays, the lack of generation of oligomannosyl *N*-glycans from labeled tri-glucosyl oligosaccharides *in vivo*, and the inability to process oligosaccharides of the VSV G protein to mature ones *in vivo* when glucosidase I and II were inhibited by castanospermine [24, 25]. Nevertheless, evidence for some residual endomannosidase activity in CHO and CHO Lec23 cells has been obtained recently. In CHO Lec23 cells, which are deficient for glucosidase I activity, structural analysis revealed presence of some complex *N*-glycans [17]. Furthermore, in these cells polysialic acid was demonstrated on a small fraction of N-CAM, which requires the previous synthesis of complex *N*-glycans [17]. In our recent studies [21], we also observed some processing of *N*-glycans of wild-type and of Z-variant of α 1-antitrypsin stably expressed in CHO cells under castanospermine-imposed glucosidase blockade.

We report here the cloning of endomannosidase from CHO cells and establish a molecular basis for the endomannosidase deficiency in CHO cells. We find that the highly conserved Arg177 and Trp188 are both substituted by Cys in CHO cell endomannosidase. However, only the Trp188Cys substitution in CHO

endomannosidase results in its mislocalization to the ER and greatly reduced *in vivo* activity.

Materials and methods

Reagents and antibodies. Polyclonal rabbit anti-rat endomannosidase antibody was kindly provided by Dr. R. G. Spiro (Boston, MA) and rabbit anti-human Sec61 β by Dr. B. Dobberstein (Heidelberg, Germany). Rabbit anti-human α 1-antitrypsin antibody was purchased from Dako (Zug, Switzerland), mouse monoclonal anti-myc antibody from Upstate (Milton Keynes, UK), rhodamine-conjugated (Fab')₂ fragments of goat anti-rabbit IgG and Alexa 488-conjugated (Fab')₂ fragments of goat anti-mouse IgG from Molecular Probes (Eugene, OR) and horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Restriction enzymes, T4 DNA ligase, alkaline phosphatase, endoglycosidase H (Endo H), recombinant *N*-glycosidase F (PNGase F) and protease inhibitor tablets were purchased from Roche Diagnostics (Rotkreuz, Switzerland), the reverse transcription system from Promega (Wallisellen, Switzerland), Taq DNA polymerase, expression vectors pcDNA3.1 and pcDNA6/myc-His, pGEM-T easy vector, competent *E. coli* DH5 α cells, Lipofectamine 2000, cell culture media and fetal bovine serum from Invitrogen (Basel, Switzerland), TRI reagent from Lucerna Chem (Luzern, Switzerland), SMART RACE cDNA amplification Kit from BD Biosciences (Basel, Switzerland) and QIAquick PCR-purification Kit from QIAGEN (Basel, Switzerland). Oligonucleotides were synthesized by Microsynth (Balgach, Switzerland). [³⁵S]Methionine/cysteine was purchased from Anawa Trading SA (Wangen, Switzerland) and protein A magnetic beads from Dynal (Hamburg, Germany).

Cell lines and transfections. The CHO Lec 23 cells [17, 18] were kindly provided by Dr. Pamela Stanley (New York, NY) and cultured in minimal essential medium Eagle (alpha modification) containing 10% fetal bovine serum (FBS). CHO-K1 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in Ham's F12 medium supplemented with 10% FBS. Transfections of CHO Lec23 cells with the pcDNA3.1/ α 1-antitrypsin and the pcDNA6/endomannosidase constructs were performed using Lipofectamine 2000 according to the manufacturer's instructions. For selection, cells were grown in medium containing G418 (1 mg/ml) and Blasticidin (10 μ g/ml). Clonal cell lines were established in 96-multiwell plates and individual clones were tested for expression of α 1-antitrypsin and endomannosidase by immunofluorescence and by RT-PCR.

Cloning of endomannosidase from CHO-K1 cells. Total RNA from CHO-K1 cells was isolated, reverse transcribed and used in the subsequent PCR amplification with the following rat endomannosidase [15] primers: ratEndo (sense) 5'-GCCAAGAACTATCCCCAAGG-3'; ratEndo (antisense) 5'-CTGTTCCGGGTGTTCTGAGT-3'. The missing 5' and 3' end of the cDNA encoding CHO endomannosidase was isolated with a 5' and a 3' RACE procedure, respectively, according to the manufacturer's instructions. The full-length CHO endomannosidase was amplified by PCR using a sense primer (P1) containing a *Hind* III site and the Kozak sequence 5'-AAAAGCTTATCATGG-CAAAATTTCTGAAGA-3' and an antisense primer (P2) containing a *Xho* I site 5'-AACTCGAGTGAAGTAGGCAGCTGTTGAT-3'. The amplicon was subcloned into the *Hind* III/*Xho* I sites of the expression vector pcDNA6/myc-His, in frame with the C-terminal myc- and His-tag.

Site-directed mutagenesis in CHO cell endomannosidase and rat endomannosidase. All mutant CHO and rat endomannosidase were generated by a PCR-based site-directed mutagenesis strategy. Briefly, full-length CHO endomannosidase cDNA served as template in combination with the CHO endomannosidase Cys177Arg antisense primer which contained a *Sca* I site, (5'-GGGCCAGTACTCAATT-

GAGGCTGAGCGCATTTGTCTC-3') and the P1 primer (see above) as well as the CHO endomannosidase Cys188Trp sense primer with a *Sca* I site, (5'-ATTGGAGTACTGGCCCTTTCTGGTACCCGCCTGA-3') together with the P2 primer, respectively. The PCR-product containing the specific mutation was subcloned into the pcDNA6/CHO endomannosidase constructs, replacing the wild-type sequence. The same strategy was applied for the generation of CHO endomannosidase Cys188Ser (5'-ATTGGAGTACTGGCCCTTTCTTCTTACCCGCCTGA-3') and for Cys188Ala CHO endomannosidase (5'-ATTGGAGTACTGGCCCTTTCTGCCTACCCGCCTGA-3') in combination with primer P2. For the CHO endomannosidase Δ Cys177,188, the *Sca* I/*Xho* I fragment of CHO endomannosidase Cys188Trp was subcloned into CHO endomannosidase Cys177Arg replacing the corresponding wild-type sequence. The Trp188Cys rat endomannosidase was generated using the following primer: 5'-ATTGGAGTACTGGCCCTGTCTTGCTACCCACCTGA-3' in combination with a 3'-rat endomannosidase-specific antisense primer containing a *Xho* I restriction site.

Endomannosidase hybrid constructs. For the rat/CHO endomannosidase hybrid construct I, full-length rat endomannosidase in pcDNA6/myc-His vector [21] was digested with *Sca* I/*Xho* I. The ~900-bp fragment was replaced by the corresponding *Sca* I/*Xho* I fragment from CHO endomannosidase. The reverse procedure was applied for the CHO/rat endomannosidase hybrid construct. For the rat/CHO endomannosidase hybrid II construct, the *Nco* I/*Xho* I fragment of rat endomannosidase was replaced by the corresponding one from CHO endomannosidase.

In vivo endomannosidase assay. Endomannosidase activity was determined on the basis of the processing of the oligosaccharides of human α 1-antitrypsin. Glucosidase I-deficient CHO Lec23 cells expressing human α 1-antitrypsin and myc-tagged endomannosidase constructs were metabolically labeled with [³⁵S]methionine for 20 min and chased for different time periods. Cell lysates (IC) and culture medium (EC) were immunoprecipitated with anti-human α 1-antitrypsin antibody and the immunoprecipitates subjected to digestion by Endo H (1U) or PNGase F (1U) for 16 h at 37°C prior to 8% SDS-PAGE/fluorography as previously described [21]. Radioactivity was visualized by fluorography after treatment with EN³HANCER using X-Omatic AR film or phosphorimager (FUJI Film Corp., Japan).

Combined immunoprecipitations and Western blotting. For immunoprecipitation, microsomal extracts [21] from CHO Lec23 cells, expressing either myc-tagged rat or CHO endomannosidase were incubated overnight with anti-endomannosidase antibody magnetic beads at 4°C. For Western blot analysis, immunoprecipitates or microsomal extracts were separated in 8% SDS-polyacrylamide gels (20 μ g protein/lane), and transferred onto nitrocellulose membranes using a semidry blotting apparatus [26]. The membranes were incubated with 0.4 μ g/ml mouse monoclonal anti-myc antibody overnight at 4°C, followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibody.

For non-reducing conditions, microsomal extracts were heated for 5 min at 100°C in non-reducing sample buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue), and analyzed by SDS-PAGE on an 8% polyacrylamide gel. Western blot analysis was performed as described above.

Confocal immunofluorescence microscopy. CHO Lec23 cells stably expressing myc-tagged endomannosidase were formaldehyde-fixed and saponin-permeabilized as described [21]. For double immunofluorescence, cells were simultaneously incubated with monoclonal anti-myc antibody (4 μ g/ml) or either anti-Golgi mannosidase II or anti-Sec61 β antibodies for 2 h at ambient temperature, and washed three times with PBS containing 1% BSA, followed by incubation with the appropriate fluorescent secondary antibodies for 1 h. After rinses in buffer and double distilled water, coverslips were embedded in Moviol.

Results

Isolation of full-length cDNA encoding CHO cell endomannosidase. We have isolated and characterized the cDNA encoding endomannosidase from CHO cells using a cDNA template from CHO cells in combination with rat endomannosidase oligonucleotide primers. An amplified RT-PCR fragment of ~700 bp exhibited high homology to endomannosidase of rat (GenBank accession number: AF023657), mouse (GenBank accession number: NM_172865), human (GenBank accession number: BX640869) and *Xenopus laevis* (GenBank accession number: BC077304). Two additional PCR products of ~1000 bp and ~2600 bp were isolated by 5' and 3' RACE, which also showed high homology to known endomannosidases. With the three independent PCR products, the 3261-bp full-length cDNA encoding CHO endomannosidase could be constructed. The open reading frame encoded a 462-amino acid protein of 53 223 Da (Fig. 1), which was in agreement with deposited endomannosidase amino acid sequences from mouse, rat and human. The hydrophobicity profile [27] showed a stretch of 16 hydrophobic amino acids comprising the residues 10–25 at the N terminus (Fig. 1). The putative CHO cell endomannosidase exhibited striking sequence identity to rat (88%), mouse (88%), human (86%) and *Xenopus laevis* (65%) endomannosidase (Fig. 1). No significant primary sequence homology to other known proteins existed. Supporting evidence for authenticity of the cloned cDNA was obtained by combined immunoprecipitation/Western blot analysis of myc-tagged CHO cell and rat endomannosidase expressed in CHO Lec23 cells (Fig. 2). The ER-retained CHO cell endomannosidase was synthesized as a 53-kDa protein. However, Golgi-localized rat endomannosidase existed as a 58- and a 61-kDa species probably due to Golgi-associated post-translational modifications (Figs. 2 and 3a).

The highly conserved Arg177 and Trp188 are substituted by Cys in CHO cell endomannosidase.

Despite the striking sequence identity of the putative CHO cell endomannosidase with the other cloned endomannosidases, discrete amino acid substitutions were observed. Noteworthy, the highly conserved Arg177 and Trp188 were each substituted for Cys in CHO cell endomannosidase (Fig. 1). Other highly conserved residues such as Cys9 and 345 as well as Met23 and 24 were preserved in CHO cell endomannosidase (Fig. 1). A characteristic of the rat, mouse, human and *Xenopus laevis* endomannosidase are three consecutive Arg residues near the N terminus.

Endoman_CHO	MAKERRKTCIILSLFILFISLMGLKMLRENTSAFGTFFGLDLLPELHPPNAPLENKVD	60
Endoman_Mouse	MAKERRRTCILLSLFILFISLMGLKMLWENAASFGPPFGLDLLPELHPLNAHSGNKAD	60
Endoman_Rat	MAKERRRTCIIISLFIVFISLMGLKMLWENAASFGPPFGLDLLPELRPPNTHLENKAD	60
Endoman_Human	MAKERRRTCIIILALFILFISLMGLKMLRENTATFCAPFGLDLLPELHQRTIHLGKNFD	60
Endoman_X.laevis	MIRERRRTCITLSIFIFLVCLIMAGLKHLPENAAFGSPFGILFPGFHR-ASVLEKIPD	59
Endoman_CHO	FQKSDRINMETNTKDLKAAVETVQPPKASEVNLEELPPLNYFLHVFYYSWYGNPQFDGKY	120
Endoman_Mouse	FQSDRINMETNTKALKGAGMTVLPKASEVNLEELPPLNYFLHAFYYSWYGNPQFDGKY	120
Endoman_Rat	FQSDRIDMETNTKDLKAGVTVHPPRASEVNLEELPPLNYFVHAFYYSWYGNPQFDGKY	120
Endoman_Human	FQKSDRINSETNTKNLKSVEITMKPSKASELNLDLPPPLNNYLHVFYYSWYGNPQFDGKY	120
Endoman_X.laevis	SENHLKGNTVTEINTLPAP-----KDKVYQLDMEEFPPPNYDLHIFYYTWYGNPOFDGKY	114
Endoman_CHO	IHWNHPVLEHWDPRIAKNYPQGIHTPPDDIGSSFYPELGSYSSRDPSVIETHMRQMCSSAS	180
Endoman_Mouse	IHWNHPVLEHWDPRIAKNYPQGHSPDDIGSSFYPELGSYSSRDPSVIETHMKQMRSSAS	180
Endoman_Rat	VHWNHPVLEHWDPRIAKNYPQGRHSPDDIGSSFYPELGSYSSRDPSVIETHMKQMRSSAS	180
Endoman_Human	IHWNHPVLEHWDPRIAKNYPQGRHNPDDIGSSFYPELGSYSSRDPSVIETHMRQMRSSAS	180
Endoman_X.laevis	IHWNHPLLKHWDAKIANSPFLGKHNPPDVGANFYPELGPYSSRDPSVIDAHMKQIRSSS	174
Endoman_CHO	IGVLALSCYPPDSNDENGEATDHLVPTILDKAHKYNLKVTFHVEPYSNRNDQNMVYTKY	240
Endoman_Mouse	IGVLALSWYPPDSRDDNGEATDHLVPTILDKAHKYNLKVTFHIEPYSNRDDQNMHQNIKY	240
Endoman_Rat	IGVLALSWYPPDASDENGEATDYLVPITILDKAHKYNLKVTFHIEPYSNRDDQNMHQNIVKY	240
Endoman_Human	IGVLALSWYPPDVNDENGEPTDNLVPTILDKAHKYNLKVTFHIEPYSNRDDQNMVYKNVY	240
Endoman_X.laevis	VGVISVSWYPPGISDDNGETDDFIPSLDKAHSYGLKVNFIHIEPYRNRDDYSLRNNVY	234
Endoman_CHO	IIDKYGSHPAFYRYKTRTGHS LPMFYTYDSYITKPNVWANLLTPSGSQSVRNSPYDGLFI	300
Endoman_Mouse	IIDKYGNHPAFYRYKTRTGHS LPMFYVYDSYITKPTIWANLLTPSGSQSVRNSPYDGLFI	300
Endoman_Rat	IIDKYGNHPAFYRYKTRMGHS LPMFYTYDSYITKPKTWANLLTPSGSQSVRGSPYDGLFI	300
Endoman_Human	IIDKYGNHPAFYRYKTKTGNALPMFYVYDSYITKPEKWANLLTSGSRSI RNSPYDGLFI	300
Endoman_X.laevis	IIDKYGSHPAFYKYKTONGRTLPMFYTYDSYITPGTWANLLTSSGSQSI RNTPYDGLIFM	294
Endoman_CHO	ALLVEEKHKNDILQSGFDGIYTYFATNGFTYGSSHQNWGNLKSFCCKNNLI FIPSVGPGY	360
Endoman_Mouse	ALLVEEKHKNDILQSGFDGIYTYFATNGFTYGSSHQNNWNLKSFCCKNNLM FIPSVGPGY	360
Endoman_Rat	ALLVEEKHKYDILQSGFDGIYTYFATNGFTYGSSHQNNWNLKSFCCKNNMI FIPSVGPGY	360
Endoman_Human	ALLVEEKHKYDILQSGFDGIYTYFATNGFTYGSSHQNWASLKLFCCKNNLI FIPSVGPGY	360
Endoman_X.laevis	ALLVEEKHKHDILRGGFDGFYTYFATNGFSYGSSHQHWSSLKEFCNTNNLL FIPSVGPGY	354
Endoman_CHO	IDTSIRPWNTQNTNRNRINGKYEVLGSAALQTHPNLISITSFNEWHEGTQIEKAVPKRIT	420
Endoman_Mouse	IDTSIRPWNTQNTNRNRVNGKYEVLGSAALQTHPSLISITSFNEWHEGTQIEKAVPKRITA	420
Endoman_Rat	IDTSIRPWNTQNTNRNRINGKYEVLGSAALQTPSLISITSFNEWHEGTQIEKAVPKRITA	420
Endoman_Human	IDTSIRPWNTQNTNRNRINGKYEIGLSAALQTRPSLISITSFNEWHEGTQIEKAVPKRTS	420
Endoman_X.laevis	IDTSIRPWNFQNTNRNRINGKYETAINAALLVREKILSITSFNEWHEGTQIEAAIPKRITA	414
Endoman_CHO	TTVYLDYRPHKPSYLYLELTRKWPEKYSKERKTYALDQQLPTS	462
Endoman_Mouse	NTIYLDYRPHKPSLYLELTRKWSEKFSKERMTYALDQQQPAS	462
Endoman_Rat	NTVYLDYRPHKPSLYLELTRKWSEKYSKERMTYALDQQLPAS	462
Endoman_Human	NTVYLDYRPHKPGLYLELTRKWSEKYSKERATYALDRQLPVS	462
Endoman_X.laevis	QMKYEDYLPHPKNIYLELTRKWSEKYMKEKEHWLV-----	449

Figure 1. The conserved Arg177 and Trp188 are substituted by Cys in CHO cell endomannosidase. The deduced amino acid sequence of CHO cell endomannosidase (GenBankTM accession number DQ825405) is compared with mouse endomannosidase (accession number NM_172865), rat endomannosidase (accession number AF023657), human endomannosidase (accession number BX640869) and *Xenopus laevis* endomannosidase (accession number BC077304). Amino acids conserved in all five species are highlighted and the Cys substitution of the conserved Arg177 as well as Trp188 is marked by an asterisk. The bar above the amino acid sequence corresponds to a hydrophobic region as deduced from the Kyte-Doolittle hydrophathy plot [27].

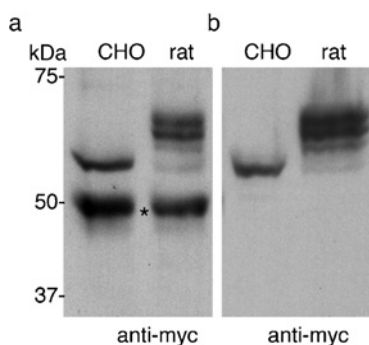


Figure 2. Expression of myc-tagged CHO cell or rat endomannosidase in CHO Lec23 cells. (a) Immunoprecipitated CHO cell or rat endomannosidase was subjected to Western blot analysis using anti-myc antibodies. The asterisk indicates position of immunoglobulin heavy chain. (b) Western blot analysis of CHO Lec 23 cell extracts expressing myc-tagged CHO cell or rat endomannosidase.

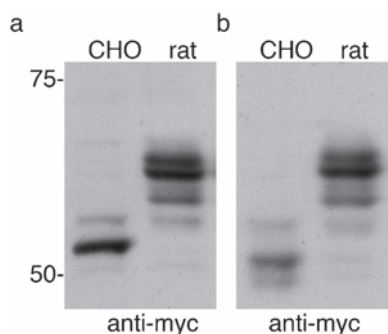


Figure 3. CHO cell endomannosidase contains an intramolecular disulfide bridge. Western blot analysis of myc-tagged CHO cell and rat endomannosidase expressed in CHO Lec23 cells under reducing conditions (a) and non-reducing conditions (b).

In CHO cell endomannosidase, two Arg residues and one Lys were found in this position.

The presence of the two additional Cys residues in CHO endomannosidase resulted in intramolecular disulfide bridge formation as indicated by differential migration behavior under reducing (Fig. 3a) and non-reducing (Fig. 3b) conditions. This was not observed for rat endomannosidase (Fig. 3a, b).

Trp188 is critical for Golgi localization and *in vivo* activity of endomannosidase. The effect of the intramolecular disulfide bridge in CHO cell endomannosidase on its subcellular distribution was analyzed by double confocal immunofluorescence. Myc-tagged rat endomannosidase expressed in CHO Lec23 cells served as a control and showed the known Golgi localization [9] as indicated by its co-distribution with Golgi mannosidase II (Fig. 4a–c). In contrast, myc-tagged CHO cell endomannosidase did not overlap with Golgi mannosidase II, but with the ER-marker Sec61 β (Fig. 4d–f). Likewise, engineered Cys177Arg/Cys188 CHO cell endomannosidase showed an ER

pattern (Fig. 4 g–i). However, engineered Cys177/Cys188Trp as well as Cys177Arg/Cys188Trp CHO cell endomannosidase exhibited Golgi localization as demonstrated by the overlap with immunofluorescence for Golgi mannosidase II (Fig. 4j–o). An ER localization was also observed for an engineered Cys177/Cys188Ser (Fig. 4p–r) and a Cys177/Cys188Ala (data not shown) CHO cell endomannosidase. Among the studied CHO cell/rat endomannosidase hybrid proteins, a Golgi localization was only observed when the rat sequence contained the Trp188 (data not shown). Furthermore, replacement of the conserved Trp188 by Cys in rat endomannosidase also resulted in ER localization (data not shown). The subcellular localization of the various endomannosidase constructs is given in Table 1.

In CHO Lec23 cells, oligosaccharides remain in an Endo H-sensitive, tri-glucosylated form because of the glucosidase I deficiency [17, 18]. Therefore, this cell line provides a suitable test system to study *in vivo* activity of endomannosidase. For this purpose, we established CHO Lec23 cells stably expressing human α 1-antitrypsin and either myc-tagged CHO cell endomannosidase or rat endomannosidase. In addition, back-mutagenized CHO cell endomannosidase as well as different CHO cell/rat endomannosidase hybrid constructs were stably expressed. The acquisition of Endo H resistance and of PNGase F sensitivity of the oligosaccharides of α 1-antitrypsin served as criterion for *in vivo* activity of endomannosidase. In glucosidase I-deficient CHO Lec23 cells, α 1-antitrypsin was synthesized as an Endo H-sensitive 54-kDa glycoprotein as expected (Fig. 5a). Endo H treatment of secreted α 1-antitrypsin resulted in one Endo H-sensitive and one Endo H-resistant species, indicating the presence of some residual endomannosidase activity (Fig. 5a). Full processing of secreted α 1-antitrypsin N-glycans to Endo H-resistant and PNGase F-sensitive ones was found in CHO Lec23 cells stably expressing rat endomannosidase (Fig. 5b). The same effect was observed when back-engineered Cys177Arg/Cys188Trp CHO cell endomannosidase was stably expressed in CHO Lec23 cells (Fig. 5c).

In further experiments we sought to establish the relative importance of the Trp188Cys and the Arg177-Cys substitution. In cells stably expressing Cys177/Cys188Trp CHO cell endomannosidase, oligosaccharides of α 1-antitrypsin became fully processed (Fig. 5d). The processing of the oligosaccharides of α 1-antitrypsin in cells expressing Cys177Arg/Cys188 CHO cell endomannosidase (Fig. 5e) was improved as compared to ordinary CHO Lec23 cells (Fig. 5a). In additional experiments, the effect of a Ser or Ala substitution at Trp188 was evaluated. In CHO Lec23 cells, expressing CHO cell endomannosidase Cys177/

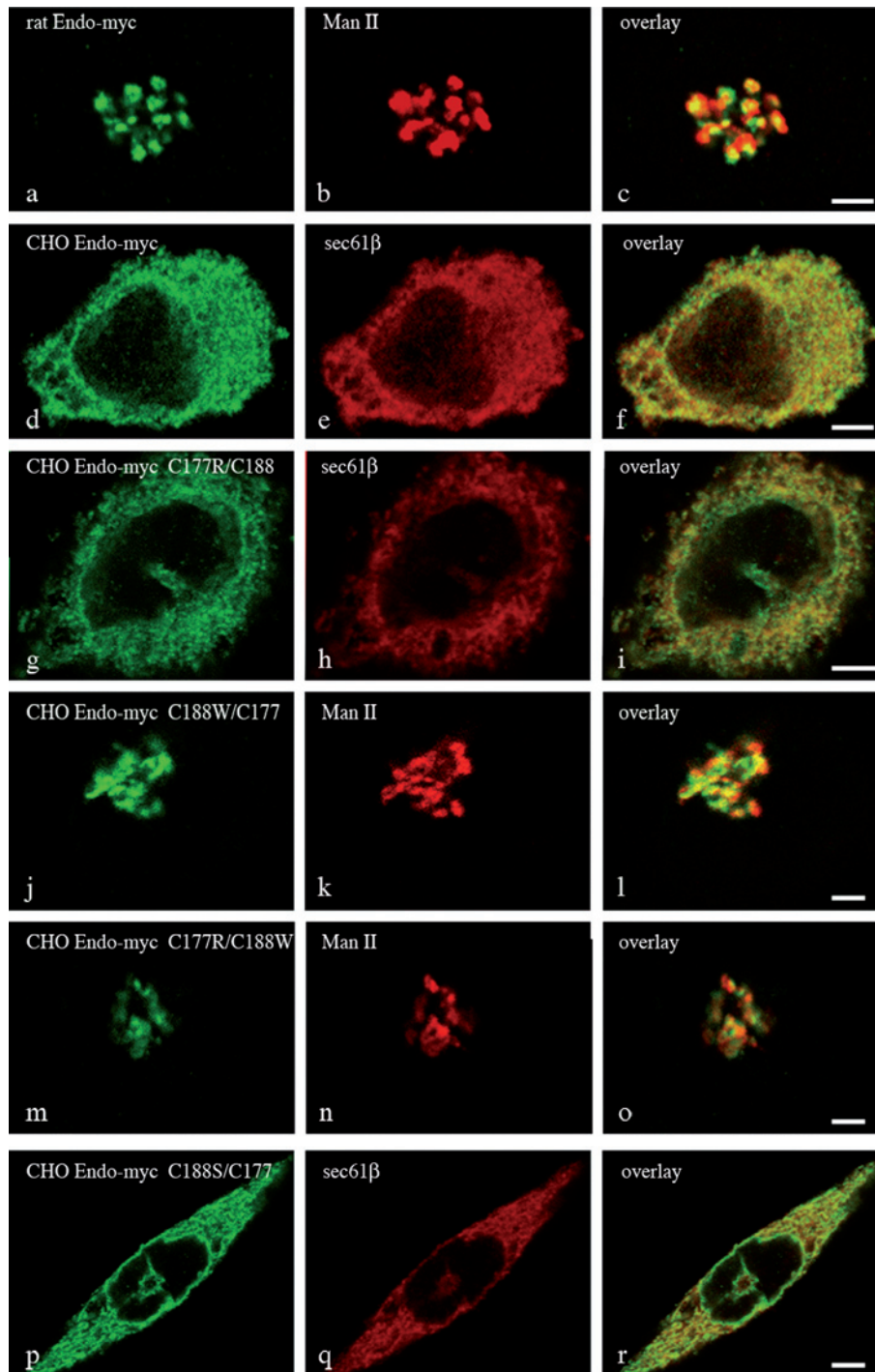


Figure 4. The conserved Trp188 in vertebrate endomannosidases is important for Golgi localization. Confocal double immunofluorescence for myc-tagged endomannosidases expressed in CHO Lec 23 cells and the Golgi marker mannosidase II as well as the ER marker Sec61 β . Rat endomannosidase (a) and Golgi mannosidase II (b) show co-distribution (c). In contrast, CHO cell endomannosidase (d) or engineered Cys177Arg/Cys188 CHO cell endomannosidase (g) overlap with Sec61 β (e, f and h, i). Golgi localization is restored in engineered Cys177/Cys188Trp (j–l) and in Cys177Arg/Cys188Trp (m–o) CHO cell endomannosidase. Engineered Cys177/Cys188Ser CHO cell endomannosidase remains ER mis-localized (p–r). Bars 5 μ m.

Cys188Ser, the processing of α 1-antitrypsin was improved as compared to that of ordinary CHO Lec23 cells (Fig. 5f), and the same was true, but to a lesser extent, for cells expressing CHO cell endomannosidase Cys177/Cys188Ala (Fig. 5g). This effect was also observed when endomannosidase hybrid constructs consisting of different portions of rat and CHO cell endomannosidase were tested (data not shown). The processing of the oligosaccharides of α 1-antitryp-

sin in CHO Lec23 cells expressing Trp188Cys rat endomannosidase was reduced (data not shown) as compared to cells expressing rat endomannosidase (Fig. 5b). All together, these data (see also Table 1) indicate that the Trp188Cys substitution in CHO cell endomannosidase or rat endomannosidase greatly diminished its *in vivo* activity.

Table 1. Localization and *in vivo* activity of various endomannosidase constructs expressed in glucosidase I-deficient CHO Lec23 cells.

Construct ^a	Localization ^b	<i>In vivo</i> activity ^c
Rat Endo-myc	Golgi	Full
CHO Endo-myc	ER	Residual
CHO Endo-myc Cys177Arg/Cys188	ER	Partial
CHO Endo-myc Cys177/ Cys188Trp	Golgi	Full
CHO Endo-myc Cys177Arg/Cys188Trp	Golgi	Full
CHO Endo-myc Cys177/Cys188Ser	ER	Partial
CHO Endo-myc Cys177/Cys188Ala	ER	Partial
Rat/CHO endomannosidase hybrid I	ER	Partial
CHO/rat endomannosidase hybrid I	Golgi	Full
Rat/CHO endomannosidase hybrid II	Golgi	Full
Rat Endo-myc Trp188/Cys	ER	Partial

^a The generation of the various endomannosidase constructs is described in the Materials and methods.

^b Localization of myc-tagged endomannosidases was performed by double confocal immunofluorescence using the Golgi marker mannosidase II as well as the ER marker Sec61 β .

^c The acquisition of Endo H resistance of the oligosaccharides of α 1-antitrypsin served as criterion for endomannosidase *in vivo* activity.

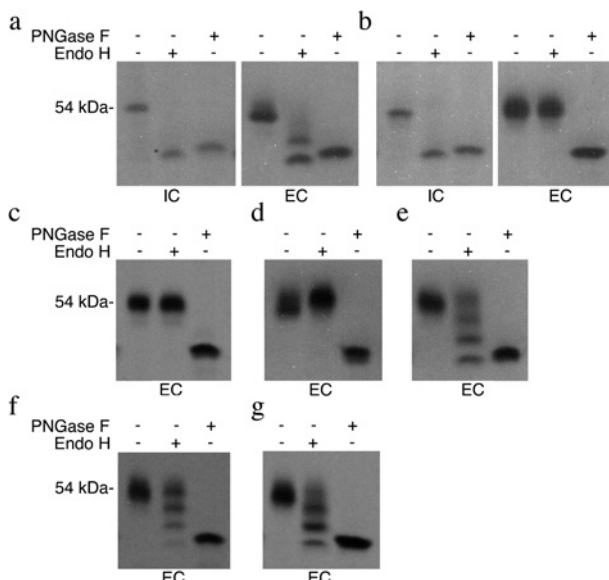


Figure 5. Engineered Cys188Trp CHO cell endomannosidase compensates for the enzyme deficiency. Processing of immunoprecipitated α 1-antitrypsin by endomannosidase from CHO Lec23 cells was analyzed (for details see Material and methods). (a) In control CHO Lec23 cells, the oligosaccharides of intracellular α 1-antitrypsin (IC) were Endo H and PNGase F sensitive. Limited processing of oligosaccharides of secreted α 1-antitrypsin has occurred as indicated by partial Endo H resistance of secreted α 1-antitrypsin (EC). (b) In CHO Lec23 cells expressing rat endomannosidase, oligosaccharides of intracellular α 1-antitrypsin (IC) were Endo H and PNGase F sensitive, whereas oligosaccharides of secreted α 1-antitrypsin (EC) were fully processed as indicated by Endo H resistance and PNGase F sensitivity. (c, d) The CHO cell endomannosidase deficiency could be rescued by expressing either engineered Cys188Trp/Cys177Arg (c) or Cys177/Cys188Trp CHO cell endomannosidase (d) as demonstrated by the Endo H resistance and PNGase F sensitivity of secreted α 1-antitrypsin. (e–g) The endomannosidase deficiency could not, however, be fully rescued by expressing engineered Cys177Arg/Cys188 (e), Cys177/Cys188Ser (f) or Cys177/Cys188Ala (g) CHO cell endomannosidase.

Discussion

In the present study, we have established the molecular basis for the endomannosidase deficiency in CHO cells. The single Trp188Cys substitution had a dual effect in that it resulted in the mislocalization of endomannosidase into the ER and in greatly reduced *in vivo* activity. Engineering and expression of a full-length CHO cell endomannosidase cDNA into Cys188Trp rescued the dual effect. Thus, the highly conserved Trp188 residue of vertebrate endomannosidases seems to be of critical importance for correct subcellular localization and *in vivo* activity.

Although Cys substitutions were found at position 177 and 188 in CHO cell endomannosidase, replacing the highly conserved Arg and Trp, only the Trp188Cys substitution appears to be functionally of relevance. This substitution resulted in intramolecular disulfide bridge formation most probably leading to a conformational change, which might account for the reduced enzyme activity. When the Cys188 was replaced by Ser or by Ala, the enzymatic activity could not be fully rescued. Thus, it is tempting to speculate that the Trp188 might be part of the substrate-binding site forming hydrogen bonds with the glucosylated oligosaccharides [6, 14, 15]. Alternatively, the Trp188 might participate in the stabilization of the tertiary structure at the active site or in the vicinity of the substrate-binding site and in that way involved in maintaining the enzymatic activity. Some experimental evidence in favor of the latter hypothesis was obtained since we observed that the Cys188Ser CHO cell endomannosidase possessed a higher processing efficiency for α 1-antitrypsin oligosaccharides than the Cys188Ala CHO cell endomannosidase.

A second consequence of the Trp188Cys substitution in CHO cell endomannosidase relates to its subcellular distribution. The observed formation of an intramolecular disulfide bridge between the Cys188 and pre-existing Cys residues might result in a conformational change. However, we obtained no evidence for the formation of endomannosidase aggregates. The mislocalization of CHO cell endomannosidase into the ER unequivocally indicates that the enzyme is transport incompetent and might be recognized and retained by the protein quality control [28]. Although inactivating point mutations have been identified for other Golgi-localized enzymes such as *N*-acetylglucosaminyltransferase I in CHO Lec1 cells [29] and BHK cells [30] or *N*-acetylglucosaminyltransferase V in CHO Lec4 cells [31], these amino acid substitutions did not affect the inherent Golgi localization of these glycosyltransferases. Conversely, the point mutation in the *N*-acetylglucosaminyltransferase V gene in CHO Lec4A cells resulted in a mislocalized but active enzyme [31]. On the other hand, a Cys residue, together with parts of the transmembrane region and the cytosolic tail, has been shown to be important for Golgi localization of an α 2,6 sialyltransferase exhibiting low catalytic activity [32, 33]. Thus, several different mechanisms occur to function together in the Golgi localization of glycosyltransferases [34]. Although the mechanistic aspects of the dual effect of the Trp188Cys substitution in CHO cell endomannosidase remain to be elucidated, most probably it is a conformational change caused by the intramolecular disulfide bridge, which makes the endomannosidase a substrate for the protein quality control. Point mutations resulting in the formation of incorrectly folded proteins are well established as the cause of many ER retention diseases [35–38].

In summary, we elucidated the molecular defect in CHO cell endomannosidase and demonstrated that the Trp188Cys substitution profoundly affects the subcellular localization and the *in vivo* activity of this Golgi enzyme.

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